Original Research Article

Investigation of Bacterial Load and Their Antimicrobial Susceptibility in an Artisanal Refining Environment

ABSTRACT

Background: Recent studies have highlighted the types of bacteria present in the atmosphere which often show predictable patterns across space and time. These patterns can be affected by presence of soot which is generated by artisanal refining and excessive burning of fossil fuel. These microorganisms are being inhaled by humans on daily basis and they also affect the properties of the bacteria such as the development of terminal spores when compared with areas with none or limited artisanal refining.
Aim: The aim of this study is to investigate the microbial load of an environment associated with artisanal refining activities.
Methodology: The samples were taken at random intervals from four different areas in a high artisanal refining state Rivers State and compared to two samples from another state Kano state both in Nigeria without artisanal refining and tested for viable bacteria load. The four amples were collected on prepared dry nutrient agar exposed to free air for a period of five (5) minutes and were covered properly and transferred to the laboratory and incubated at 37^{O}C for 24 hours. While, the two control samples were also cultured and incubated at 37^{O}C. The isolates were morphologically and biochemically determined and identified.
Results: The obtained results were gram positive rods *Bacillus spp.* and gram positive cocci *Staphylococcus spp.*
Conclusion: This work was able to identify different species of gram positive rods *Bacillus spp*. with terminal spores and gram positive cocci *Staphylococcus spp*. as bacteria associated with artisanal refining at the different sampled sites.

Key words: Bacteria, soot, artisanal, refining, fossil fuel.

1. INTRODUCTION

Artisanal refining is the process of procuring stolen crude oil and further refining them in the so-called bush refineries with the use of local resources and skills (drawing on the indigenous technology used to distil locally made gin – popularly described as ogogoro or kaikai). The basic materials typically involve rudimentary illegal skills – often metal pipes and drums welded together – in which crude oil is boiled and the resultant fumes are collected, cooled and condensed in tanks to be used locally for lighting, energy or transport [1].

Oil theft or illegal bunkering as it is known in Nigeria is an organized 'theft of crude oil from product pipelines through the use of improvised conduits and direct pumping from oil well heads into barges by criminal syndicates [2]. Boat yards help construct and supply barges to the participants to transport crude oil around the creeks. Local women supply firewood and other materials needed to workers at the camp. Many of the people involved in oil theft work for local markets, poorly refining products for communities desperate for affordable sources of energy are provided [2]. Although

the economic impact of oil theft associated with artisanal refineries has been widely reported [3], the impact of the operations of these refineries on the highly sensitive environment of the Niger Delta is scarcely reported [4].

According to Ogbuagu *et al.*, [5], it has been reported that petroleum refining contributes solid, liquid, and gaseous wastes into the environment. Some of these wastes could contain toxic components such as the polynuclear aromatic hydrocarbons (PAHs), which have been reported to be the real contaminants of oil and most abundant of the main hydrocarbons found in the crude oil mixture [6]. Once introduced into the environment, PAHs could be stable for as short as 48 hours (naphthalene) or as long as 400 days (fluoranthene) in soils [7]. They thus, resist degradation and, remain persistent in sediments and when in organisms, could accumulate in adipose tissues and further transferred up the trophic chain or web. Different studies [8, 9] opines that acute exposures to aromatic hydrocarbons, which are common constituents of oil, are known to cause respiratory symptoms and high molecular weight PAHs are of significant concern because of the mutagenicity, carcinogenicity and bioaccumulation in organic tissues due to their lipophilic character [10].

Gasoline is a complex mixture of hydrocarbons and other chemical compounds used as fuel for spark-ignition internal combustion engines, primarily in light duty transportation vehicles. Gasoline is in high demand in developing countries because of an increase in population, with a resultant increase in vehicular and industrial activities.

Furthermore, refineries are producing at below installed capacities or are not functioning at all, which has resulted in the inability to refine enough gasoline to meet local consumption. Artisanal refining activity in the Niger Delta is increasing [11]. In artisanal refining, crude oil is boiled at atmospheric temperature; the resultant fumes are condensed and collected in tanks and used locally as automotive fuel. This local refining skill is believed to have been drawn from indigenous technology [12]. The artisanal refineries operating in the creeks of the Niger Delta, though illegal, provide employment to the locals as well as bridge the gaps in the availability and supply of refined petroleum products in the oil-bearing communities of the region [12, 13]. Nigerian National Petroleum Company (NNPC) in its report stated that Nigeria is not currently refining crude oil and therefore the corporation distributes only imported petroleum products in the country.

Though gasoline produced by artisan refiners is not tested well enough to certify its compliance to any local or international set parameters; it still cushions the effect of gasoline scarcity. Makeshift techniques are used by artisan refiners in processing the raw crude oil, via thermal cracking, into useful products. These procedures could be unsophisticated and not very safe however, it could be effective.

The petroleum fractions obtained by local refiners are skeptically referred to as “bunkering oil” or adulterated products. Indigenous innovation and ingenuity in harnessing our natural resources should be appreciated, regulated, and the products assessed if they meet local and international specifications. Also, there is a need to assess the level of quality compliance of the gasoline samples distributed in the area to guard against environmental pollution and engine malfunctioning.

According to Vempatapu & Kanaujia [14], physicochemical properties like distillation profile, research octane number (RON), motor octane number (MON), and Reid vapor pressure are frequently used to detect the adulteration and quality of gasoline. It is on this basis that this research was designed to compare the physicochemical properties of regular automotive gasoline and locally refined gasoline. This study aims to investigate the presence of microbes available in artisanal refining areas with its associated soot.

2. MATERIALS AND METHODS

2.1 Study Location

The study area is in Port Harcourt metropolis of Rivers State. It is a coastal city located in the Niger Delta region of Nigeria. It lies on latitudes 04º45º and 55º North and Longitude 06º and 70º50º East, with a land mass of 109,966 square kilometers which is about 0.95% of Rivers States land mass of 10,432,281 square kilometers. The air samples were collected from four different points that were identified using the Geographical Positioning System (GPS) in a State with high incidence of artisanal refining activities Rivers State in Nigeria. The samples were collected from four locations identified as Ojoto Roundabout (Lat:4.78^{O}, Lng:6.99^{O}), Nembe Waterside (Lat:4.75^{O}, Lng:7.03^{O}), Rivers State University Teaching Hospital (Lat: 4.77^{O}, Lng:7.05^{O}), and Rumuokalagbor Village (Lat:4.81^{O}, Lng:7.01^{O}), all in Port Harcourt, Rivers State and compared to two areas without such activities in Kano State, No. 33 Lamido Crescent and God is Good Motors Park, Kano State, all in Nigeria. The choice of these two states is as a result of the high incidence of artisanal refining and a state not undergoing such processes and their maps are shown in **figure 1** and **2** below. Locations were exposed to nutrient agar during inoculation.

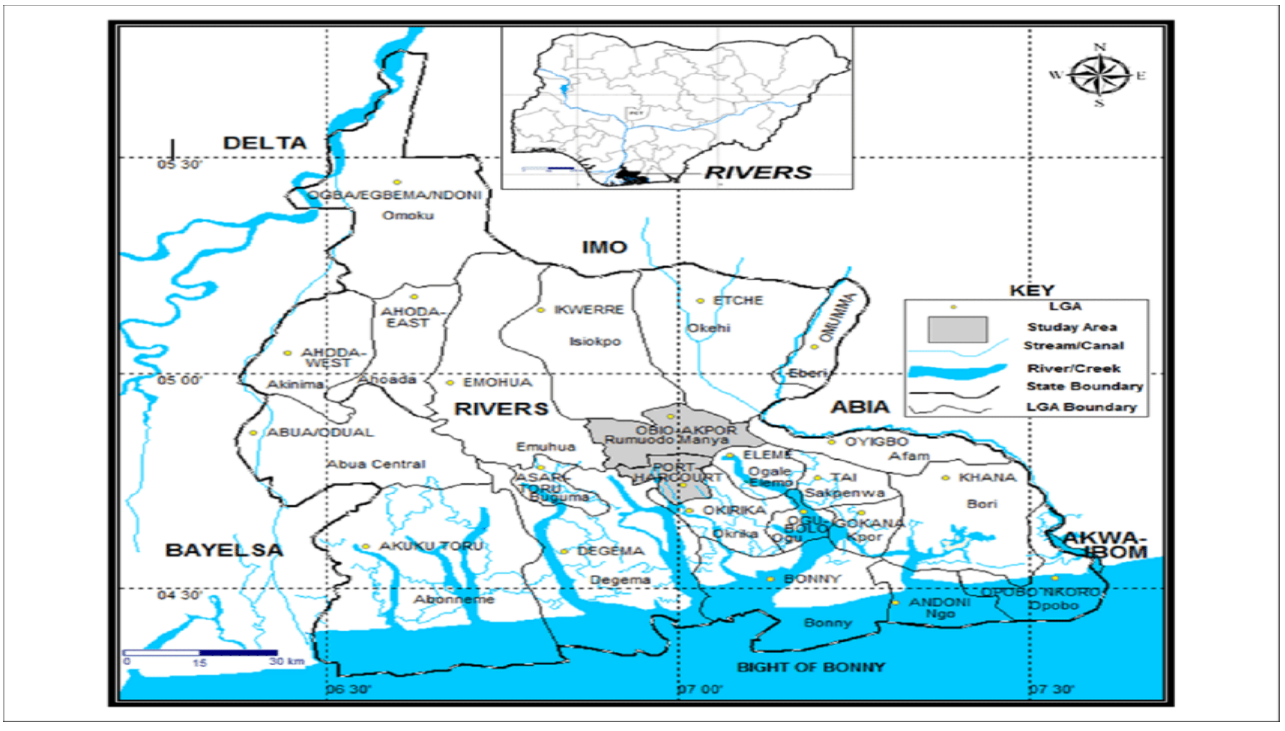

Figure: 1 Map of Rivers State showing the position of Port Harcourt City.

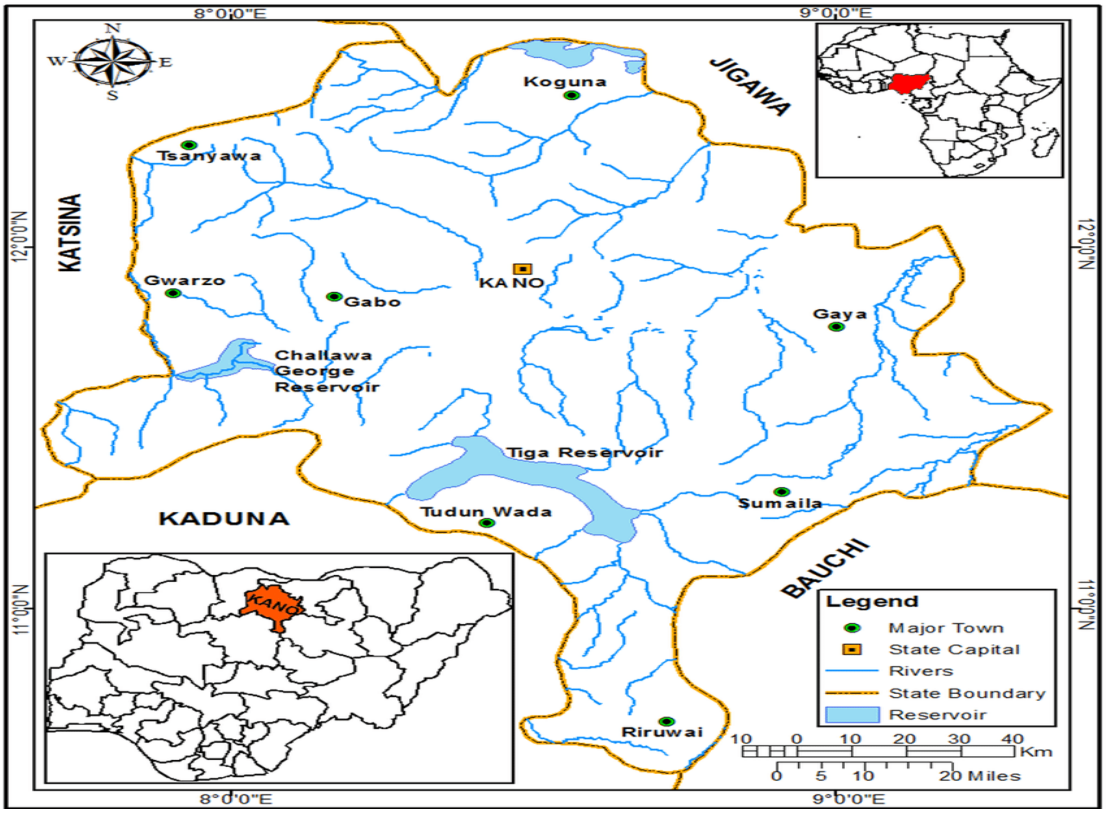

Figure: 2 Showing the Map of Kano State.

2.2 Sample Collection and Processing

The experimental sample used in this research was air. Air samples were collected at Ojoto round about, Rivers State University Environ, Nembe Waterside, Rivers State University Teaching Hospital (RSUTH), Rumuokalagbor Village, Rivers State University Microbiology Laboratory, Mile 1 Park all in Port Harcourt, Rivers State and No. 33 Lamido Crescent Kano State and GiG Park, Kano State, all in Nigeria. The different locations were chosen to ascertain the microbial content in the atmosphere.

2.3 Media Preparation

2.3.1 Nutrient Agar Preparation

The nutrient agar plate was prepared according to the manufacturer's specification by weighing out the required grams of the powder and dissolving it in the required volume of water. The mixture was heated and stirred to fully dissolve all components. The dissolved mixture was autoclaved at 121^{O}C for 15 minutes, this was allowed to cool, poured into sterile petri dishes and allowed to solidify.

2.4 Sample Inoculation

Sterile nutrient agar plates were taken to the four identified locations and two control areas randomly and differently, close or far from artisanal activities and exposed to air for 5 minutes and were carefully closed and transported in an air tight cold chain bag to avoid contamination before incubation in an incubator for 24 hours at 37^{O}C.

The plates were checked for bacteria growth the next day and they were counted and recorded. The isolates were sub-cultured on sterile Nutrient agar plates and were used for biochemical tests and bacteria identification.

2.5 Biochemical Test

2.5.1 Gram Stain

Gram-staining method is most important procedure in microbiology. The different procedure separates most bacteria into groups of basic cell wall composition. Gram positive bacteria (thick layer of peptidoglycan, 90% of cell wall) stains purple while gram negative bacterial (thin layer of peptidoglycan, 10% of cell wall and high lipid content) stains red/pink.

Procedure: Gram stain was done for each of the bacteria isolated. A drop of normal saline was placed on a grease free glass slide. With the aid of a wire loop, a small amount of the colony was picked and emulsified in normal saline. The smear was allowed to air dry. The slides were placed on the staining rack and flooded with the primary stain which is crystal violet, allowed for 60 seconds and rinsed in water. Lugol's Iodine was added and left for 60 seconds then rinsed in water. Rapid decolourization was done using ethanol and rinsed in water. It was counter stained with Safranin and allowed for 60 seconds then rinsed with water. The slides were allowed to air dry and examined microscopically using the immersion oil.

2.5.2 Catalase Test

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacteria isolate is evident when an inoculum is introduced into hydrogen peroxide and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

Procedure: With the aid of a wire loop a small amount of colony growth was transferred onto the surface of a clean, dry glass slide. A drop of 3% H_2O_2 was dropped on the glass slide it was then observed for the evolution of oxygen bubbles.

2.5.3 Coagulase Test

Coagulase is an enzyme-like protein and causes plasma to clot by converting fibrin to fibrinogen. *Staphylococcus aureus* produces two forms of coagulase: bound or free. Bound coagulase (Clumping factor) is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in an alternation of fibrinogen so that it precipitates on the staphylococcal cell, causing the cell to clump when a bacterial suspension is mixed with plasma. This does not require coagulase reacting factor. Free coagulase involves use of plasma coagulase reacting factor (CRP), which is modified or derived from thrombin molecule to form a coagulase CRP complex. This complex in turn reacts with fibrinogen to produce the fibrin clot.

Procedure: The slide method was used. A drop of normal saline was placed on a clean grease free glass slide. Sterile wire loop was used to pick a colony and emulsified. A drop of fresh plasma was added and then mixed thoroughly. The mixture was observed for agglutination microscopically. To confirm the presence or

absence of agglutination, the mixture is covered with cover slip and examined under the microscope.

2.5.4 Motility Testing

Hanging drop method was used to show organisms that are motile. A ring of plasticine was applied on a clean slide. A loopful of overnight broth culture of the organism was placed in the centre of a coverslip. The ring of plasticine was carefully pressed on the cover-slip with the drip of culture in the center of the ring and not touching the slide. With a quick movement, the slide was inverted so that the cover-slip is uppermost. The preparation was examined microscopically using x10 and x100 objective.

2.6 Statistical Analysis

Data obtained from the study were analyzed using Microsoft Excel Package, 2019.

2.7 Antimicrobial Sensitivity Test (Kirby-Bauer disk diffusion method)

A bacterial suspension equivalent to 0.5 Mcfarland standard was prepared by picking 2 colonies from the pure culture and dissolving in physiological saline solution. The suspension was spread platted using a sterilized spreader on nutrient agar. Antimicrobial impregnated disk CPX, NB, CN, AMX LEV, S, E, RD, CH, APX were placed on the culture medium surface using a sterilized forceps. The plates were incubated at 37^{O}C for 24 hours. After incubation, the antimicrobial's efficacy was determined by measuring the diameter of zones of inhibition and interpreted. The bacterial strains were classified as Susceptible (S) or Resistance (R) using the Clinical and Laboratory Standard Institute (CLSI) technique.

Table:1 Showing the Various Antibiotics used, their Strength and Classes

Antibiotics	Strength	Class of antibiotics
Ciprofloxacin	10 µg	Fluoroquinolones
Norfloxacin	10 µg	Quinolones
Gentamycin	10 µg	Aminoglycosides
Amoxil	20 µg	Penicillin
Streptomycin	30 µg	Aminoglycosides
Rifampicin	30 µg	Macrolides
Erythromycin	30 µg	Macrolides
Chloramphenicol	30 µg	Macrolides
Ampiclox	20 µg	Penicillin
Levofloxacin	20 µg	Fluoroquinolones

3. RESULTS

The total Heterotrophic Bacteria Count for the different locations is shown in table 2 from the use of sterile nutrient agar plates in the areas where soot particles were found as the bacteria were isolated and numbers of colonies determined. The Total Heterotrophic Count shown in table 3**,** indicates that samples from Rumuokalagbor village have a high number of bacteria growth colonies with a colony forming unit of 1.43×10^{6} while sample from Rivers State University Teaching Hospital had lesser colony forming unit of 7.5×10^{5}, even as soot particles were usually found on the

benches indoor and the free air we breathe. However, the Total Heterotrophic Bacteria Count from our control is seen to be very low with 3.2×10^5 and 2.8×10^5 respectively.

Table: 2 Showing Total Heterotrophic Bacteria Count at the different Locations

Location of isolates	Number of Colonies	Colony Forming Unit (cfu/ml)
Ojoto round about	88	8.8×10^5
Nembe Waterside	80	8.0×10^5
Rumuokalagbor Village	143	1.43×10^6
R.S.U.T.H	75	7.5×10^5
Control		
No 33 Lamido Crescent, Kano State	32	3.2×10^5
GiG Park, Kano State	28	2.8×10^5

Key:
RSUTH= Rivers State University Teaching Hospital.
RSU= Rivers State University.
GiG= God is Good Motors.

The table:3 shows the identified bacteria from the different locations and the Gram staining results. Microorganisms such as *Staphylococcus aureus, Bacillus spp*. and *Staphylococcus spp.* were identified from various locations. Few isolates were gotten from the entire laboratory with a total of 22 isolates, 18 *Bacillus spp.* (77), 3 *Staphylococcus spp*. (18%) and *1 Staphylococcus aureus* (5%) as also seen in figure 3 whereas table 4 shows the catalase, coagulase and motility tests results carried out on the isolates. The antimicrobial sensitivity results are seen in table 5 with Ciprofloxacin (77%) having higher sensitivity followed by Levofloxacin (66.6%). Norfloxacin (0%), Rifampicin (0%) and Ampiclox (0%) were seen to be highly resistant to the microorganisms. The figure 4 on the other hand shows the percentage of the isolates to the antimicrobial gents.

Table 3: Shows identified Bacteria from the different locations

Location	Suspected Organism
Rivers State University Teaching Hospital	*Staphylococcus spp.*
	Bacillus spp.
Rumuokalagbor Village	*Staphylococcus spp.*
Nembe Waterside	*Bacillus spp.*
Ojoto Junction	*Bacillus spp.*
Control	
No 33 Lamido Crescent, Kano State	*Bacillus spp.*
GiG Park, Kano State	*Bacillus spp.*

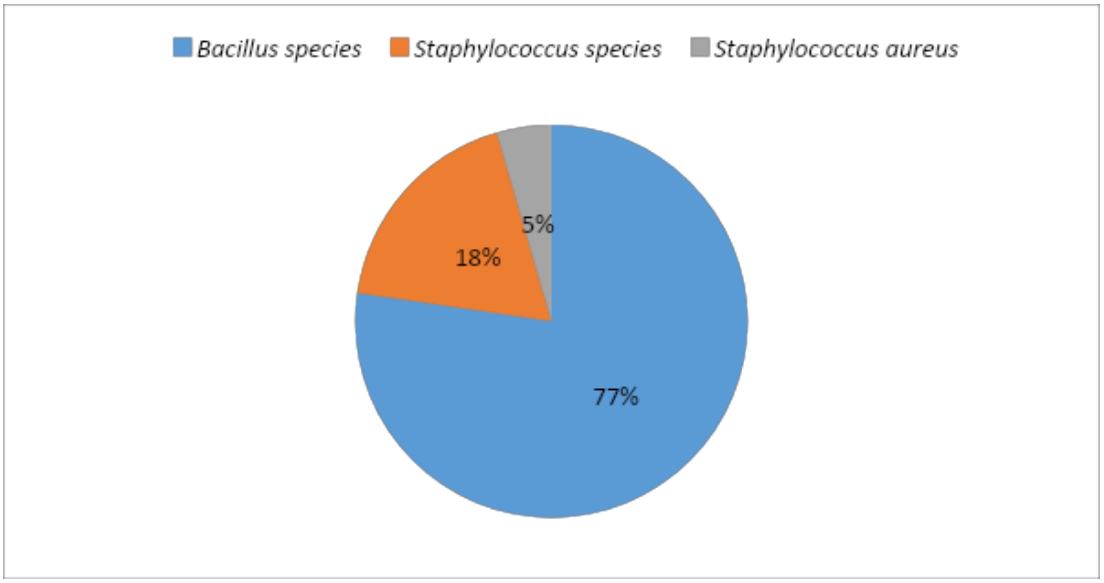

Figure 3: Shows the Percentage distribution of the Total Bacteria isolated

Table 4: Biochemical Test carried out

	Catalase	Coagulase	Motility test
Positive	22(100%)	2(9%)	18(81.8%)
Negative	0(0%)	20(90.9%)	4 (18.2%)

Table 5: Shows the Reaction Percentage of the Isolates to the Antimicrobial Agents

Isolate	Ciprofloxacin (CPX)	Norfloxacin	Gentamycin (CN)	Streptomycin (S)	Rifampicin (RD)	Erythromycin (E)	Chloramphenicol	Amplicox	Levofloxacin
1st	S	R	R	S	R	R	S	R	S
2nd	S	R	R	S	R	S	R	R	R
9th	R	R	R	R	R	R	R	R	R
10th	S	R	R	R	R	R	R	R	S
11th	S	R	R	R	R	S	R	R	R
13th	S	R	S	R	R	S	S	R	S
14th	S	R	R	S	R	S	S	R	S
17th	S	R	R	S	R	R	R	R	S
18th	S	R	S	S	R	S	R	R	S
	77.7%	0%	22.2%	55%	0%	55%	33.3%	0%	66.6%

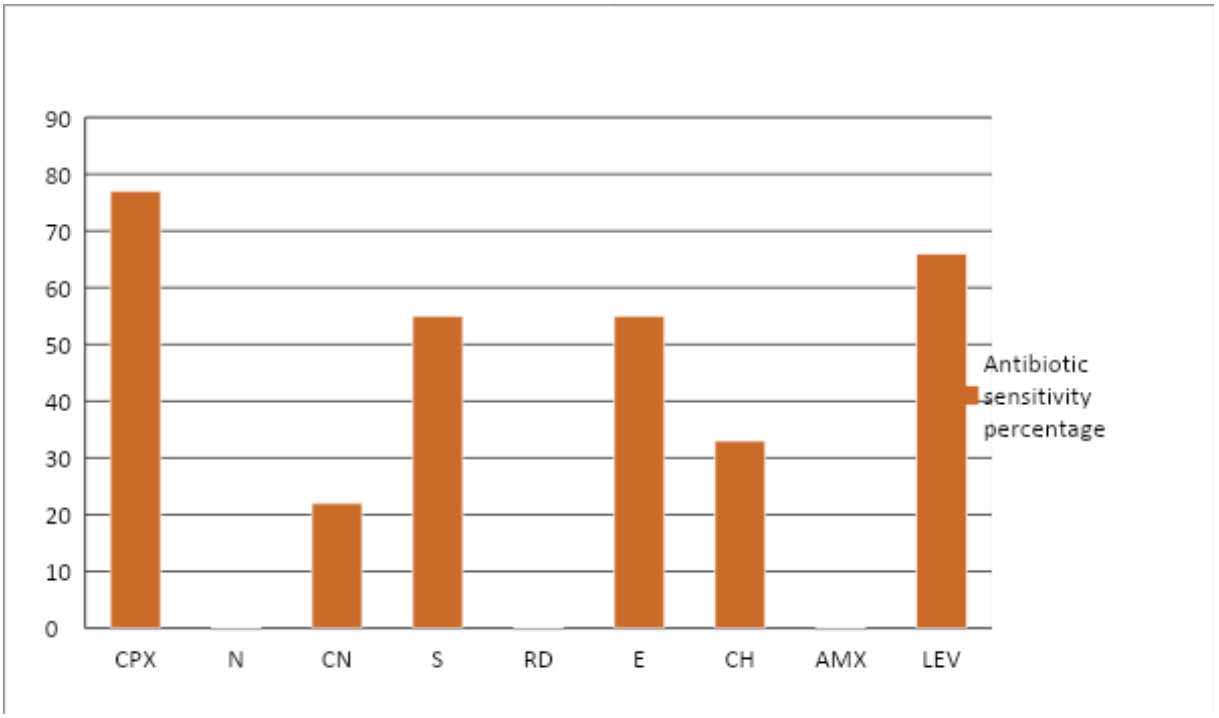

Figure 4: Shows the Reaction Percentage of the Isolates to the Antimicrobial Agents in Bar Chart.

4. DISCUSSION

Microorganisms such as bacteria are ubiquitous in nature and are common contaminants in our environment. These microorganisms are affected by the condition of the environment. If the environment is harsh or difficult for the survival of microorganism, they will have to adapt to survive. In this study, it was observed that most bacilli found in the atmosphere in which artisanal refining take place develop spores, most at the terminal end. It was also found that there was an increase in the number of microorganisms in the air. This could have adverse effects on humans living in this geographical location due to the inhalation of such organisms. If the menace of soot is not controlled in the nearest future it might lead to a spike in certain new diseases which could be cardiovascular related.

New researches suggests that air pollution may have an effect on human health by altering the bacteria genetic makeup. It shows that hydrocarbon, a major component of crude oil that causes air pollution, dramatically changes the genetic and phenotypic composition of bacteria, which can affect their survival in the lining of airways and their resistance to antibiotics [15].

This study has revealed the high presence of microorganisms in the soot impacted adjoining environment from artisanal refineries in the Niger Delta region. The high pollution index reported for the impacted ecosystems indicates a negative effect of the artisanal activities in the environmental matrix. The prevalence of hydrocarbon

degraders in the environmental matrix strongly suggests that the environment have been chronically polluted by petroleum hydrocarbon.

Results from this present study have shown that from the total number of six samples consisting of four for artisanal refining and two from areas without collected from the environment (air) , a total of 22 isolates were obtained:18 (77%) were *Bacillus species*, 3 (18%) were *Staphylococcus species* and 1 (5%) was *Staphylococcus aureus* (figure 3). However, it was observed that air samples collected from areas with artisanal refining activities had more microbial growth and developed terminal spores which aid their survival in air as seen in table 3.

Staphylococcus aureus are usually found in the nose, skin or throat of all healthy individuals, therefore the presence of the bacterium in air could be because of transfer of human aerosol probably from coughing, sneezing, talking and other similar mechanism. This high volume of soot has also been seen to help the development of these bacteria in air.

Morrissey *et al*., [16] opined that the effects of hydrocarbon on bacteria, "organisms central to ecosystems in humans and in the natural environment, are poorly investigated or studied". It is therefore important for scientist to do more ground breaking study on how soot affects the microbial world.

The reaction of the isolates exposed to some antimicrobials have also shown that these bacteria can still react to some commonly available antimicrobials if they become infective in humans. Although the presence of spores by the organisms could bring about the problem of resistance.

5. Conclusion

The presence of soot caused by artisanal refining in Port Harcourt, Rivers State, Nigeria causes an increase in bacterial growth and development of spores that causes an increase in the lifespan of the bacteria and is considered not to be safe for humans in addition to various possible cardiovascular illnesses. These organisms include: bacteria isolates; *Bacillus sp.,* and *Staphylococcus sp.* Therefore, strict implementation on stopping artisanal refining in our communities is recommended. This could go a long way in reducing public health risk posed by such exposures to the air we breathe. Further prolonged study should be conducted using molecular based techniques for further identification of the organisms present in air samples. The Government should put policies in place to stop artisanal refining activities which increases the presence of soot.

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